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Direct carrier detection by in situ suppression hybridization with cosmid clones of the Duchenne/Becker muscular dystrophy locus

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Summary. A basic problem in genetic counseling of families with Duchenne/Becker muscular dystrophy (DMD/BMD) concerns the carrier status of female relatives of an affected male. In about 60% of these patients, deletions of one or more exons of the dystrophin gene can be identified. These deletions preferentially include exon 45, which can be detected by multiplex polymerase chain reaction (PCR) and Southern blot analysis of genomic cosmid clones that map to this critical region. As a new approach for definitive carrier detection, we have performed chromosomal in situ suppression (CISS) hybridization with these cosmid clones in female relatives of four unrelated patients. In normal females, most metaphases showed signals on both X chromosomes, whereas only one X chromosome was labeled in carriers. Our results demonstrate that CISS hybridization can define the carrier status in female relatives of DMD patients exhibiting a deletion in the dystrophin gene.

Introduction

The gene responsible for Duchenne/Becker muscular dystrophy (DMD/BMD) maps to Xp21 and is the largest human gene known so far. At least 65 exons coding for an mRNA of 14 kb are distributed within a region of 2.3 Mb (for review see Love and Davies 1989). Recently, the coded protein, called dystrophin, has been identified. It is localized at the cytoplasmic face of the plasma membrane of the muscle fibers. In DMD patients, no dystrophin can be detected in muscle tissue using antibodies against defined regions of dystrophin (Hoffman et al. 1987; Koenig et al. 1988; Bonilla et al. 1988), whereas BMD patients usually show an altered molecular weight of dystrophin (Arahata et al. 1989).

Within the dystrophin gene deletions that lead to the disease phenotype can be detected in about 60% of DMD patients (Koenig et al. 1989; Den Dunnen et al. 1989). Approximately 60% of these deletions include exon 45 and occur in the region defined by the probe P20 (Wapenaar et al. 1988).

About one third of all DMD patients arise by new mutations. In the remainder, the mutation has occurred in one of the germ cells that formed the zygote in an earlier generation. This situation results in a high carrier risk for the mother and other female relatives of an affected boy (Davie and Emery 1978).

In addition to clinical investigations (for review see Moser 1984), there are several biochemical and molecular approaches for carrier detection. All these approaches have their limitations (see Discussion). We have developed a direct approach that enables us to define unequivocally the carrier status of female relatives from patients exhibiting a deletion. This approach is based on the site-specific hybridization of cosmid clones using chromosomal in situ suppression (CISS) hybridization (Landegent et al. 1987; Lichter et al. 1990; Kievits et al. 1990). Here, we used three cosmid clones spanning the region around exon 45 (Fig. 1). Using a clone containing a DNA region that is deleted in the patient, carriers are expected to show a signal in Xp21 only on one of their X chromosomes, whereas in non-carriers both of the X chromosomes should exhibit a hybridization signal.

Families and methods

Families

Four possible carriers (probands) of the dystrophin gene were investigated in four independent families A, B, C and D (Fig. 2). In each of these families, the diagnosis of DMD or BMD was confirmed by the presence of a DMD-cDNA deletion detected by a multiplex PCR analysis in the affected boys. In family A, the patient (A3) expressed a dystrophin of reduced molecular weight supporting the diagnosis of BMD, in agreement with a mild clinical course of the disease. Dystrophin was absent in the patient (C3) of family C, indicating DMD. D4 is a BMD patient with a dystrophin

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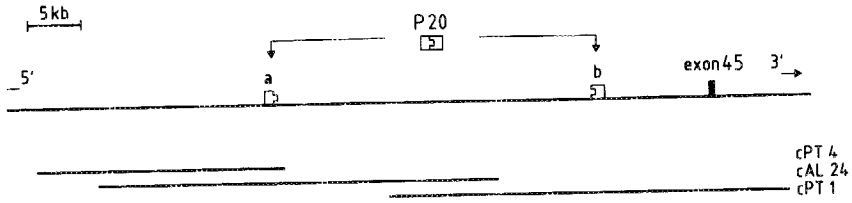

Fig. 1. Localization of cosmid clones *cPT1*, *cAL24* and *cPT4* in the *P 20* subregion of the DMD gene (for further details see Blonden et al. 1989)

of reduced weight and abundance. In the patient (B8) of family B, dystrophin was not investigated. In this family, DMD was evident by the clinical course and pedigree analysis. Three healthy non-related women not carrying a detectable dystrophin gene deletion and an obligate carrier of family B (B4, as indicated by pedigree analysis) served as controls.

Methods

Multiplex PCR-analysis. The nine primer pairs described by Chamberlain et al. (1989) and an additional primer pair (Speer et al. 1989) were used for amplification of 200 ng of template DNA according to the protocol of Chamberlain et al. (1989) with the following modifications: 25 cycles using 2.5 units CETUS *Taq*-polymerase were performed and no DMSO was added. Amplification products were separated on a 3% NuSieve agarose gel (Seakem) in 1 × TBE buffer.

DNA-Probes. DNA from the three cosmid clones cPT1, cAL24, cPT4 (Blonden et al. 1989, see also Fig. 1) and from the plasmid pXBR containing human X centromer-specific alphoid repeat (Yang et al. 1982) were prepared according to standard procedures (Maniatis et al. 1982). Probes were labeled by nick-translation substituting dTTP by Biotin-11-dUTP (Sigma) as described by Langer et al. (1981). A phage DNA library established from sorted human X chromosomes was purchased from ATTC (catalog no.: 57750) and labeled by nick-translation with Digoxigenin-11-dUTP (Boehringer Mannheim, FRG).

Cosmid hybridization of Southern-blots. Digestion of human genomic DNA with *Hin*dIII, gel electrophoresis and Southern blotting were performed following standard procedures. Labeling of cosmid probe DNA with random priming and competitive hybridization were carried out as described (Blonden et al. 1989).

Chromosome preparation and CISS-hybridization. Metaphase spreads from PHA-stimulated human lymphocytes were fixed with acetic acid/methanol (1:3 v/v) and stored until use at −80°C. CISS-hybridization of biotinylated cosmid clones was carried out as described by Lichter et al. (1990). In experiments using a single cosmid clone, 200 ng labeled cosmid DNA, 2 µg sonicated human placenta DNA for competition and 8 µg sonicated salmon sperm DNA (Sigma) were added to 10 µl hybridization mixture (sufficient for an area of 18 × 18 mm) containing 50% formamide (Fluka), 10% dextran sulfate and 1 × SSC. In other experiments, all three cosmid clones were combined using 100 ng of each labeled probe. When co-hybridization with pXBR was performed, 20 ng probe DNA in 1 µl hybridization mixture were added to the cosmid probe after the preannealing step (Cremer et al. 1988). Hybridized probes were detected with fluorescein-conjugated avidin (Vector Laboratories). The signal was amplified once using a biotinylated anti-avidin antibody (Vector Laboratories) and subsequent detection with fluorescein-conjugated avidin (Pinkel et al. 1986). For co-hybridization of the whole X-library, 400 ng digoxogenin-labeled total phage DNA from this library and 100 ng of each biotinylated cosmid was used. The signals were detected with a TRITC-conjugated anti-digoxigenin antibody (Boehringer Mannheim) and the subsequent detection of the cosmid clones using avidin-conjugated to FITC and one amplification step (see above). Fluorescence microscopy was performed with a Zeiss Photomikroskop III equipped for epifluorescence. Photographs were taken on an Agfachrome 1000 RS color slide film.

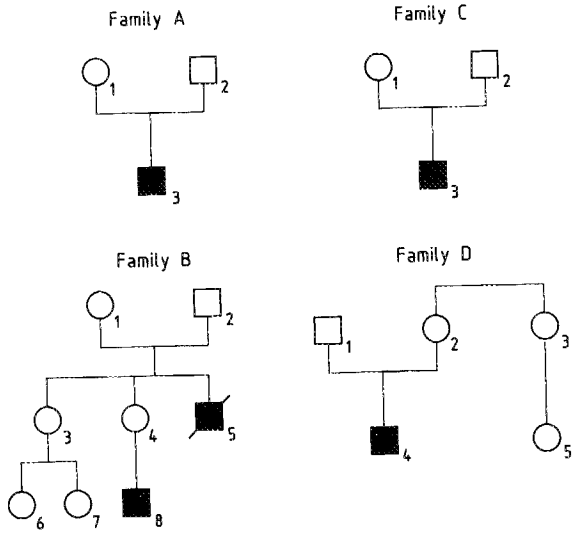

Fig. 2. Pedigrees of families A, B, C and D

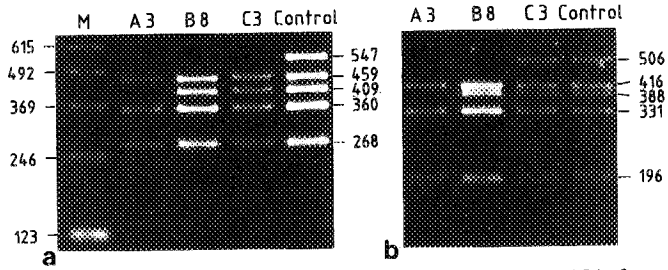

Fig. 3a, b. Multiplex DNA amplification of genomic DNA from patients *A3*, *B8* and *C3*. Amplification products in **a** represent exons 45 (547 bp-band), 49 (459 bp), 51 (409 bp), 8 (360 bp) and 44 (268 bp) of the dystrophin gene, and in **b** represent exons 48 (506 bp), 17 (416 bp), 51 (388 bp), 12 (331 bp) and 4 (196 bp). *Patient A3* presents a deletion of the dystrophin gene from exon 45 to at least exon 52, *patient B8* from exon 45 to 51 and *patient C3* only for exon 45. As a *control*, DNA from a normal male was amplified. We used a 123-bp ladder *(M)* as size marker

Results

The DNA of patients A3, B8 and C3 (Fig. 2), screened for a deletion using the multiplex PCR-primer system (Fig. 3), shows the loss of exon 45 of the dystrophin gene. To determine whether the deletion around exon 45 extends at least the length of the cosmid cPT1 (Fig. 1),

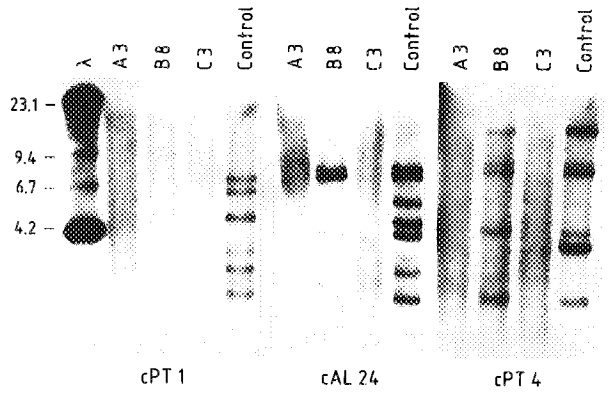

Fig. 4. Southern-blot hybridization of *Hin*dIII-digested DNA of DMD/BMD patients with the cosmid clones *cPT1*, *cAL24* and *cPT4*. The normal *control* shows the expected bands. *Patients A3* and *C3* are deleted for all three cosmids, whereas *patient B8* is completely deleted for cPT1 and partially for *cAL24* and *cPT4*

Southern blots from these patients were hybridized with the cosmids cPT1, cAL24 and cPT4 (Fig. 4). In patients A3 and C3, all three cosmid clones were deleted. In patient B8, only cPT1 was completely deleted, whereas the two other clones were partially deleted (Fig. 4). Based on these results, CISS-hybridization of metaphase spreads from the obligate carrier B4 and the possible carrier B3 were performed with the cPT1 clone, whereas the possible carriers A2 and C2 in the other two families were investigated by CISS-hybridization with either cPT1 or the pooled clones cPT1, cAL24 and cPT4, in order to improve the hybridization signal.

CISS-hybridization of the single or pooled cosmid clones to metaphase spreads from healthy non-related female individuals from three non-affected families resulted in specific signals on both X chromosomes in most metaphase spreads (Fig. 5a, Table 1).

As expected, in the obligate carrier B4, only on X chromosome was labeled by cPT1 in all metaphase spreads that exhibited specific signals with this probe. There was no case of a metaphase spread exhibiting label on both X chromosomes (Fig. 5b, Table 1). The co-hybridization of the repetitive centromere probe pXBR (Yang et al. 1982) was used to facilitate the identification of the deleted X chromosome (Fig. 5b). The same result was seen in her sister B3 (with a 50% a priori risk of being a carrier) and demonstrates unequivocally that she carries the same deletion (Table 1). This result thus enables the direct investigation of the carrier status of the daughters of B3 by the method of CISS hybridization. Moreover, CISS-hybridization with the pooled cosmids performed in the probands A2 and C2 also resulted in the specific labeling of one X chromosome only, establishing that both probands are carriers of the deleted X chromosome (Table 1).

After the investigation of the described three families had been completed, family D was included in this study. Patient D4 showed a deletion of exons 45–48 by PCR reaction and a complete deletion of the cPT1 clone in Southern blot hybridization (data not shown). Using samples from his mother D2, a possible carrier, we performed cohybridization with an X-chromosome-specific library labeled with digoxigenin for rapid identification of the X chromosomes and the biotinylated cPT1 cosmid clone. D2 showed hybridization signals with cPT1 on both X chromosomes on metaphase spreads (Fig. 5c,d); Table 1) and in nuclei (Fig. 5e, f), suggesting that she does not carry the DMD mutation (for the possibility of germline mosaicism, see Discussion). Proband D3 (Fig. 2), however, referred to us for genetic counseling, could definitely be excluded as a carrier for this mutation (see Discussion).

Discussion

Because of the high proportion of new mutations, which are estimated to be about 30% in DMD/BMD patients, genetic counseling of female relatives provides a particular problem when the carrier status cannot be deduced by pedigree analysis alone. The usefulness of the present biochemical and molecular approaches of carrier detection is limited. An elevation of the level of creatine kinase (CK) in carriers was first described by Schapira et al. (1960). However, since only 70% of adult carriers have an elevated CK level, a normal CK value does not exclude carrierships (Moser 1984). Intragenic restriction fragment length polymorphisms (RFLPs) to identify the X chromosome segregating with the DMD mutation may be misleading because of possible recombinations between the site of a DNA marker and the mutation site (Bakker et al. 1986). In sporadic cases, the carriership of the mother as studied by RFLP analysis may be considered unlikely if a second healthy son carries the same X chromosome as his affected brother. Such an interpretation, however, may also be misleading because of the possibility of germline mosaicism (Bakker et al. 1989; Darras et al. 1988). In cases with a defined deletion, gene dosis analysis in Southern blots from maternal DNA provides a further possibility for carrier detection (Mao and Cremer 1989). Under routine laboratory conditions, however, it may be problematic to base the decision of the carrier status of a female relative solely on this type of analysis. A definite carrier diagnosis can also be achieved by the identification of size altered fragments detected by field inversion gel electrophoresis (FIGE), but may not yield unequivocal results if the deletions are small (Chen et al. 1988). In DMD families, immunostaining of muscle tissue by dystrophin antibodies has been successfully used only for the detection of carriers with elevated CK levels. In BMD families, the usefulness of immunostaining or immunoblotting for carrier detection has not been established so far (Arahata et al. 1989; Miranda et al. 1989).

In this study, we have demonstrated that CISS-hybridization of cosmid probes specific for a deleted subregion of a patient's dystrophin-gene can be used to investigate directly the presence of this subregion on one or both X chromosomes of female relatives. In cases where only one X chromosome shows a hybridization signal, this approach provides unequivocal evidence confirming the carrier status of these females, even if the CK or RFLP analysis is not informative.

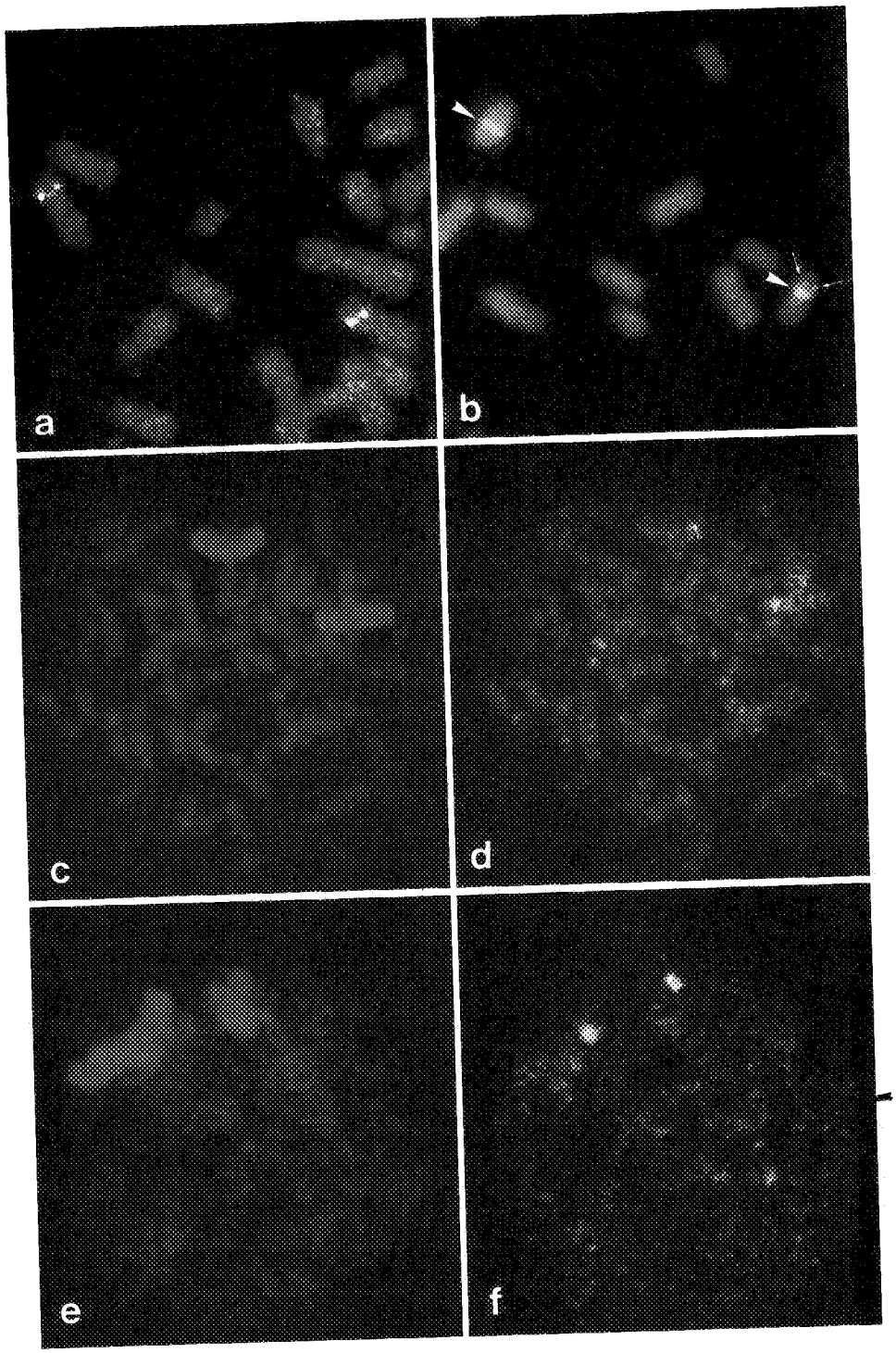

Fig. 5a–f. CISS hybridization of DMD-specific cosmid clones. **a** Metaphase spread of a normal female hybridized with the biotinylated cosmid cPT1 and counterstained with propidium iodide. On both X chromosomes, fluorescence signals are seen on both chromatids of the short arm. ×2000. **b** Metaphase spread of an obligate carrier of family B (B4) after co-hybridization of the centromere-specific probe pXBR and cPT1. Chromosomes are counterstained with propidium iodide. The centromeres of both X chromosomes are delineated with pXBR *(arrowheads)*. The cosmid signals on Xp are seen on one X chromosome *(arrows)* but not on the other. × 1500. **c, d** Metaphase spread of a possible carrier D1 after double hybridization with an X-chromosome-specific library and cPT1. Both X chromosomes are delineated using a filter for rhodamine fluorescence (**c**). The cosmid signal is clearly seen on both X chromosomes with a filter for fluorescein. Even with this filter, the X chromosomes can be detected (**d**). × 2000. **e, f** Hybridization signal on a prophase nuclei of the same patient. Rhodamine filter (**e**) and fluorescein filter (**f**). × 1400

In cases where both X chromosomes contained in the somatic cells of a female relative show hybridization signals, the interpretation is more complicated because of the repeated observation of germline mosaicisms in the mother (Bakker et al. 1989; Darras et al. 1988). Accordingly, such a finding does not rule out unequivocally a recurrence risk for further pregnancies of the mother, and prenatal diagnosis should still be offered. For the same reason, sisters of the patients may possibly be carriers and should be investigated independently of the outcome in the mother. On the other hand, a normal result in the somatic cells of a patient's mother would be sufficient to rule out the possibility of a carriership in her sisters.

Our approach is limited by the fact that deletions in the dystrophin gene can be detected so far in only 60% of the patients. In the families presented in this investigation, a deletion spanned at least the length of one cosmid clone, i.e., about 40 kb. The progress of non-radioactive in situ hybridization techniques has made it possible to detect single-copy targets as small as 2–5 kb (Lichter et al. 1988; Cherif et al. 1989). Accordingly, all iden-

Table 1. Evaluation of hybridization efficiency with the DMD-specific cosmid probes (only cPT1 or three cosmids as a pool). From each proband expect D1 (50), 100 metaphases were counted

Proband	Probe	N	Hybridization signal		
			Both X	One X	None
A1	Pool	100	0	93	7
B3	cPT1	100	0	94	6
B4	cPT1	100	0	91	9
C1	Pool	100	0	92	8
D1	cPT1	50	43	1	6
Normal	Pool	100	85	2	13
Females	Pool	100	91	1	8
	cPT1	100	89	2	9

tifiable deletions more than 2 kb in size in the dystrophin gene should be accessible to this technique provided that the necessary clones become available for in situ hybridization. In families where the affected males have already deceased, in situ hybridization of probes defining deletion-prone subregions of the dystrophin gene may be directly used to screen potential carriers. Such an approach will become even more powerful following the development of multi color in situ hybridization techniques that allow the simultaneous staining of multiple gene targets in different colors (Nederlof et al. 1990). We expect that, in addition to the analysis of X chromosomes in metaphase spreads, DMD carrier detection will become possible in future by the analysis of multicolored hybridization signals directly in interphase nuclei.

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